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$associatedsarcomas.$

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A RETROSPECTIVE ANALYSIS OF RADIATION THERAPY FOR THE TREATMENT OF FELINE VACCINE-ASSOCIATED SARCOMA *

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ABSTRACT

We retrospectively evaluated predictive prognostic factors in 73 cats with vaccine-associated sarcoma given postsurgical curative (n=46, most with clean margins) or coarse fractionated radiotherapy (n=27, most with either macroscopic disease or dirty margins). The former animals displayed a median survival of 43 months and a median progression free interval (PFI) of 37 months, the latter reached a median survival of 24 months and a median PFI of 10 months. In cats undergoing coarse fractionated therapy, factors predictive of a better outcome included lack of visible mass (n=10) as opposed to macroscopic disease (n=17, survival: 30 vs. 7 months, P=0.025; PFI: 20 vs. 4 months, P=0.01), adjuvant chemotherapy for gross disease (n=5/17, survival: 29 vs. 5 months, P=0.04) and a smaller number of surgeries preceding radiation therapy (Coeff=0.41, P=0.03). The Ki67-index was not predictive for survival. We concluded that postsurgical curative and coarse fractionated radiotherapy are both effective legitimate options for managing vaccine-associated sarcomas.

KEY WORDS

Feline vaccine-associated sarcoma, curative radiotherapy, coarse fractionated radiotherapy, Ki67-index

*This study was conducted in the context of a doctoral thesis (C Eckstein)

INTRODUCTION

In the US cat population feline vaccine-associated sarcomas have an incidence of approximately 1/10'000[1-3] to 1/1000[4] cats. One study in Germany estimated the incidence in this country to one case per 1000 cats[5]. Vaccine-associated sarcomas account for about 40% of all feline skin tumours[6-8] and are the most frequent skin tumour in this species[5].

Although the pathogenesis of vaccine-associated sarcoma has not been definitively elucidated, it is believed to involve chronic local inflammation, which has been associated with the adjuvant in the vaccines[1,2,9,10] and age-related immunodeficiency[11]. In addition, there have also been single reports of sarcoma development after the injection of other agents like methylprednisolone or penicillin[12], and lufenuron[13]; even suture material was associated with the formation of fibrosarcomas[14].

Vaccine-associated sarcomas are highly invasive and often rapidly growing neoplasms. The evolution of treatment from single[15-17] to multimodality[18-21] therapy has resulted in longer progression free interval (PFI) and survival time. For example, in cats treated with surgery only the median survival time was 576 days[17], in cats treated with chemotherapy only there was a 50% overall response rate and the median survival of the responders was 242 days[16]. In contrast, in cats undergoing multimodality treatment, radiation therapy prior to surgery resulted in a median survival time of 600 days[20], and when radiation therapy was applied after surgery the median survival time was 730 days[18]. But despite the combination of aggressive surgery and curative radiation therapy, treatment still fails in many patients. For this reason and because of its high

costs and time requirements, curative radiation therapy is not always an acceptable option for the owners. A coarse fractionation protocol was designed as an alternative. To the authors' knowledge such a protocol has not been published so far for the therapy of vaccine-associated sarcomas.

Vaccine-associated sarcomas have histologic characteristics considered either unique[53] or suggestive for this diagnosis[22] as opposed to sarcomas not associated with vaccination. In addition to a broad spectrum of histologic types, they usually display peripheral inflammation consisting predominantly of lymphocytes and smaller number of plasma cells usually located around blood vessels[23]. The presence of peripheral aggregates of macrophages containing intracytoplasmic globular grey–brown material, which has been shown to contain aluminium, supports the diagnosis of vaccine-associated sarcoma[24].

Both, histologic features of soft tissue sarcomas and tumour proliferation index have been examined in human and animal tumours. High tumour proliferation has been shown to correlate with aggressive behaviour in soft tissue sarcomas in humans[25-29]. Among several different possibilities to assess cell proliferation, the Ki67-index has been proposed as an intrinsic cell kinetic parameter with potential prognostic value in human soft tissue sarcomas[25-29] and in canine tumours of various origins[30-35, 47]. In cats the Ki67-index was determined in apocrine sweat gland tumours[36], melanocytic tumours[37], squamous cell carcinomas[38], lymphoma[39] and mammary tumours[40]. One paper reports determination of the Ki67-index in feline vaccine-associated sarcomas[41]. No significant differences were found among the average proliferation rates determined

through measurements of the Ki67 reactivity at the tumour periphery among tumours of different histologic grade[41]. In addition, no significant difference was found between the proliferation index at the periphery and in central areas of the tumours. To our knowledge, the prognostic value of the Ki67-index has not yet been investigated in feline vaccine-associated sarcomas. Immunohistochemistry for Ki67 is well established for formalin fixed, paraffin-embedded tissues[41,42]. Therefore, it is well suited to perform retrospective studies.

The two major aims of this retrospective study were 1) to evaluate the survival and PFI of curative versus coarse fractionated radiotherapy patients and 2) to determine the growth fraction of vaccine-associated sarcomas by Ki67 immunohistochemistry and the predictive value of the Ki67 labelling index. Additional variables assessed in the outcome analysis included demographical and clinical characteristics as well as histological parameters.

MATERIAL & METHODS

Patients

Medical records of all cats with histologically confirmed soft tissue sarcomas that underwent radiation therapy at the section of Diagnostic Imaging and Radiation Oncology at the Vetsuisse Faculty, University of Zurich, Switzerland between 1996 and 2005 were reviewed. Only cats in which the tumour was determined to be vaccine-associated on the basis of vaccination history and tumour location were included in the study. Thus, cats with sarcomas in locations not associated with vaccination (i.e. head, tail, oral cavity, distal regions of the limbs) were excluded.

The diagnostic work up in the selected cats included a physical examination, a complete blood count and a chemistry panel to evaluate the general health status, and two lateral thoracic radiographs to search for distant metastases. Additional laboratory and diagnostic tests, as well as further imaging procedures were conducted as needed. Tumour location, presence and size of gross tumour or of a scar, as well as the number of previous excisions were gathered from the medical records for statistical analysis.

Radiation Therapy

All cats were treated with external beam megavoltage radiation. Radiation was delivered with a linear accelerator (Dynaray LA20, VARIAN, Zug, Switzerland) using 9-16 MeV electrons. Treatment plans were calculated manually and the field dimensions were adjusted to enclose at least a 3 cm margin of presumed normal tissue adjacent to the tumour or the scar. Dose distribution was improved by using tissue-

equivalent bolus material. Therapy plans were designed to maximally spare the spinal cord as well as the thoracic and abdominal organs.

Irradiation was delivered with either a curative intent or a coarse fractionation protocol. The prescribed total dose for curative radiation therapy was either 48 Gy (12 x 4.0 Gy, delivered on a Monday-Tuesday-Thursday-Friday schedule) or 45 Gy (9 x 5.0 Gy, delivered on a Monday-Wednesday-Friday schedule). The standard protocol for coarse fractionation consisted of 4 fractions of 8.0 Gy each administered once a week. Criteria shifting decision towards the use of the coarse fractionation protocol included the presence of gross, nonresectable tumour, incomplete tumour excision, and concurrent medical problems. Surgical excision was considered “wide” if there was >1 cm of non-neoplastic tissue around the tumour as determined by histological examination. If tumour cells did not extend to the excision margin, yet were within 1 cm of the margin, tumour excision was then called “clean but close”. In cases with either “wide” or “clean but close” margins, excision was considered as “complete” and for these cats curative radiation therapy was proposed to the cat owner. When neoplastic cells extended to the cut surface the margins were considered “dirty” and surgical excision was considered "incomplete". In these cats a coarse fractionation protocol was recommended. However, the final decision on which protocol to follow was left to the owner, which entails heterogeneity in the 2 treatment groups due to deviations from our recommendations. In some cases the margins had not been evaluated. These excisions were classified as “not assessable” and the decision towards one of the protocols was made on other variables, i.e. age, health, size of the scar, etc. Chemotherapy was recommended for all cats receiving coarse fractionated radiotherapy.

For radiation therapy, cats were anesthetized with either propofol (Propofol 1% Fresenius®, Fresenius Kabi AG, Stans, Switzerland) administered as the sole anaesthetic agent, or a combination of propofol/midazolam (Dormicum®, Roche Pharma AG, Reinach, Switzerland) or midazolam/ketamin (Narketan®10, Vetoquinol AG, Belp, Switzerland) administered to effect. Oxygen was provided by mask during radiation.

Follow-up evaluation

During the recheck examination three weeks after completion of radiotherapy, patients were assessed for acute side effects. Thereafter, the owners were instructed to consult for assessment of response to therapy at three-month intervals. These rechecks included regular thoracic radiography to evaluate for gross pulmonary metastasis. For cats that were not re-evaluated at the Vetsuisse Faculty, around the time of data analysis (September 20th 2005) the referring veterinarians and owners were queried by telephone interview about general health and tumour status and in case of death, if the cause was tumour-related or unrelated. No necropsies were performed.

Progression free interval (PFI) was defined as the time from the start of radiotherapy at the Vetsuisse Faculty in Zurich to the onset of local recurrence or progression of gross disease or development of distant metastasis. Survival time was defined as the time between the start of radiotherapy and the date of death or the date of last follow-up or the date of data analysis for animals still alive. In animals with gross disease, response to treatment was defined as follows: a “complete response” was defined as disappearance of all measurable disease based on physical examination. “Partial

remission" indicated a tumour size reduction by ≥50%, with no new lesions developing. "Stable disease" was defined as less than 50% size decrease or up to 25% increase, and "progressive disease" indicated an increase in tumour measurements of ≥25%, or the development of new lesions or metastasis. At the occurrence of progressive disease, additional therapy was offered to all patients, regardless of the initial treatment. The options offered included either chemotherapy, a second round of radiation therapy, ancillary surgery or a combination of these modalities.

Histopathological reassessment

Paraffin blocks of the cases were obtained when available either from the archives of the Institute of Veterinary Pathology Zurich or from external diagnostic labs. Histopathology was performed (by FG and CE) using hematoxylin and eosin-stained sections. Depending on the predominant cellular morphology the tumours were classified either as fibrocytic (indicating a spindle-cell morphology), histiocytic or mixed cell type, referring to a mixed fibrocytic-histiocytic variant (Figure 1). Tumour grade based on cellular differentiation, presence and extension of necrosis within the neoplasm, and mitotic rate was assessed as described by Couto *et al.*[41] (Table 1). This scheme was originally designed for the grading of soft tissue sarcomas in humans[29] and later adapted for veterinary medicine by Kuntz *et al.*[44]

Tissue array construction and immunohistochemistry

The paraffin blocks obtained were used for tissue array construction. During histopathological analysis, areas with viable tumour tissue and areas with necrosis or with marked inflammatory infiltrates were labelled on each corresponding tissue

section. Labelling was used to select tumour regions in the paraffin blocks devoid of necrosis and marked inflammation for tissue array construction. Tissue cores (0.6 mm in diameter) were taken randomly from the preselected tumour areas; the number of cores taken per tumour ranged from 6 to 50, with a mean of 22. In a first step, between 6 and 12 cores were taken depending on the cellular density of each particular tumour tissue. Further cores were added later as needed in order to reach the minimal number of cells required for the Ki67 counts. Sections from the arrays were labelled immunohistochemically with antibodies specific for T or B lymphocytes and for Ki67.

CD3 and CD79alpha: Immunohistochemistry with antibodies anti-CD3 (for T lymphocytes) and anti-CD79alpha (for B lymphocytes) was used to exclude tissue areas with marked inflammatory infiltrates for the subsequent Ki67 assessment. This was done to avoid erroneous counting of lymphatic cell nuclei, which was deemed to be an issue especially in tumour cores with a transversal plane of section. Hence, only areas with less than 10% positive cells for CD3 or CD79alpha were used to determine the numbers of Ki67 positive cells. For detection of CD3 positive cells a polyclonal anti-human pan T-cell antibody (Dako Cytomation, Zug, Switzerland; Cat. Number A0452) was used. CD79alpha positive cells were labelled using a monoclonal anti-human pan B-cell antibody (Dako; Cat. Number M7051). In both cases sections were pretreated for 20 min. in a pressure cooker at 98 °C in basic buffer (pH=8; Dako; Cat. number S2367). Endogenous peroxidase was inactivated by immersing the slides in peroxidase blocking solution (Dako) for 10 min at room temperature. The anti-CD3 antibody was diluted 1:250, the anti-CD79alpha antibody was diluted 1:300, both antibodies were incubated for 1 h at room temperature. For detection of the primary antibody, the Detection Kit

(Dako) was applied according to the manufacturer's instructions. The reaction was visualized by means of an AEC chromogen (Dako) and the slides were counterstained with hemalum. A lymph node from a cat was used as a positive control, for negative controls incubation of the primary antibody was omitted.

Ki67-index: The sections were labelled for Ki67 (monoclonal antibody anti-human Ki67, clone MIB-1; Dako, cat. number M7240) using a protocol previously described for cat tissues by Melzer *et al.*[38] Positive and negative tissues included into the arrays comprised cores bearing a squamous cell carcinoma and normal skin including hair follicles and *Musculi arrectores pilorum*. The percentage of positive tumour cells was determined by computer-assisted manual counting. All slides were scanned with a ScanScope (Aperio Technologies, Inc., Vista, CA, USA) and snapshots of randomly chosen regions of each core were taken at 40x magnification using the computer program "Aperio ImageScope" version 7.1.32.1024 (Aperio Technologies, Inc., Vista, CA, USA). A sufficient number of fields were photographed to allow a minimum of 700 neoplastic cells per tumour to be counted. A minimum number of six cores were assessed for each tumour. The number of cells counted per field varied depending on the cellular density of each tumour (Figure 1). Additional cores were added when needed to reach the minimal number of cells to be counted as indicated above. The Ki67-index was defined as the percentage of Ki67 positive tumour cells and was determined by dividing the number of positive cells by the total number of positive and negative tumour cells, multiplied by 100.

Statistical analysis

To calculate the progression free interval (PFI) local recurrence, progression of gross disease and distant metastasis were defined as event. Animals that did not experience a PFI event by the time of data analysis or at last follow-up were censored. In the survival analysis deaths attributable to, or likely attributable to disease progression were considered events. Cats still alive at the time of data evaluation, or deceased due to a tumour-unrelated cause, or lost to follow-up were censored in the survival analysis. Median PFI and survival were compared with respect to demographical and clinical characteristics (i.e. age, sex, breed, weight, vaccination history, tumour location, radiation protocol, total dose (Gy), tumour margins, number of surgeries before radiation therapy, adjuvant chemotherapy, response after initial therapy) and histological parameters (i.e. cell type, grade, mitotic rate, amount of necrosis in the tumour, overall differentiation of the tumour tissue, presence of multinucleated giant cells, percentage of Ki67-positive cells) by the Kaplan-Meier method together with logrank (Mantel-Cox) and Breslow-Gehan-Wilcoxon tests and univariate proportional hazards analysis. Due to the low number of grade I tumours in this study, for statistical analysis it was necessary to dichotomize the cases into a low grade group (histological grade I and II) and a high grade group (histological grade III). In order to develop multiple Cox-regression models, predictors with a $P<0.1$ in the univariate analysis or otherwise relevant were included in the backward Cox-regression. The resulting final multiple models were as follows. For the evaluation of Ki67 in association with survival and PFI, three different predictors were considered: first Ki67 was examined in a continuous form, second a binary (0/1) variable with a cut-off by the median (10% positive cells) was computed and third a binary (0/1) variable with a cut-off of 20%

(positive cells) suggested by the ROC analysis was evaluated. The influence of the different tumour and patient characteristics as listed above on descriptors of proliferation was evaluated by using the Wilcoxon rank test and the Fisher's exact test. For statistical analysis StatView 5.0.1 (SAS Institute Inc., Cary, North Carolina, USA) and SPSS 13.0. (SPSS Inc., Chicago, Illinois, USA) were used. Throughout the study, the results of statistical analysis with P-value smaller than 5% were considered to be significant.

RESULTS

Demographics

Of the 73 cats included in this study, 29 (40%) were neutered males and 44 (60%) were spayed females. The age of the cats ranged from 4.0 to 18.0 years, with both a mean and median age of 9.0 years. Breeds represented included 66 Domestic Short Hairs, four Persian, one Main Coon, one Oriental Shorthair, and one Turkish Angora. Weight ranged from 3.0 to 7.9 kg with a mean of 4.9 kg. All 73 cats were vaccinated at least once in their life against feline viral panleukopenia/rhinothracheitis/calicivirus, and/or FeLV, and/or rabies. Primary tumour location included the neck/scapular/interscapular region (59%), the thoracic and abdominal wall (23%), or the flank/lumbar region (18%). All 73 patients were followed up to death or the time of data analysis except for one cat lost to follow-up. Thirty-two patients died or were euthanized due to tumour-related disease, 24 due to tumour-unrelated disease (i.e. car accident, heart failure, pulmonary edema, pleural effusion, liver tumour, renal failure, diabetes mellitus, ileus, ascites, etc.), and in three cases the cause of death could not be assessed. Metastases were not observed in any of the cats during or after therapy.

Radiation Therapy

In this and in the following paragraphs, details pertaining to clinical characteristics and to therapeutic modalities of the cats examined in the study are given. The most important information is summarized in Table 2.

A total of 46 cats received curative radiation therapy. In three of these cats there was a visible mass. Response was complete in one cat, which underwent surgery after radiation therapy. Stable disease was observed in the other two cats. A total of 27 cats

were treated with the coarse fractionation protocol. The majority (17/27; 63%) of these cats presented with macroscopic disease. Of those 17 patients, two had a complete response, which allowed post-radiation surgical excision, 7 cats had a partial remission, 7 cats had stable disease and one cat had progressive disease during radiation therapy.

Adverse effects of radiation were reported to be mild and self-limiting with only dry desquamation, alopecia and depigmentation of the irradiated field. Negligible differences in intensity of side effects between curatively treated cats and those receiving coarse fractionated therapy were found, but the former animals appeared to have side effects more frequently. There were no differences in the adverse effects between cats that received concurrent chemotherapy and those that had radiation therapy as a sole treatment modality.

Surgery

Of all 46 cats receiving curative radiation therapy, 45 cats had one (n=24), two (n=16), three (n=4) or four (n=1) surgeries prior to radiation. Two of these cats already developed tumour recurrence before radiation therapy had started and these cats were therefore irradiated in the gross disease setting. In one of these cats tumour margins were dirty and in the other cat tumour margins were not assessable. A third cat underwent surgery after radiation therapy; the tumour was completely excised. Altogether, tumour excision was complete in 33/46 (72%) cases (margins were wide in 22 cats and clean but close in 11 cats) and incomplete excision was found in 6/46 (13%) animals. In the remaining 5 cases (11%) completeness of excision was not assessable.

Of all 27 cats treated with the coarse fractionation protocol, 10 cats had prior surgery and were free of gross disease at the time of irradiation, and two cats irradiated in the gross disease setting underwent surgery after radiation therapy. Of the 10 cats that had surgery before radiation therapy, four had one surgery, three had two surgeries, one had three surgeries and two animals had four surgeries prior to radiation therapy. Margins were dirty in 7 out of these ten (70%) cats, clean but close in one (10%), and not assessable in two (20%) animals. In the two cats with surgery after radiotherapy, margins were wide in one cat and clean but close in the other.

Chemotherapy

Only one of the curatively treated cats received chemotherapy. It consisted of doxorubicin and was administered concurrently to radiation therapy. Altogether 13 of the 27 irradiated cats treated with a coarse fractionation protocol received additional chemotherapy. In 11 of these cats chemotherapy was started together with radiation and in the remaining two cats it was started after radiation ended. This group comprised 8 out of 10 animals with no visible mass and 5 out of 17 cats with macroscopic disease. In all cases doxorubicin (20 mg/m^2 or 1 mg/kg administered in three sessions, except for two cats that received it only once, and one cat that received it 6 times) was used as a single agent, except in one case where cyclophosphamide (250 mg/m^2 evenly distributed over four days) was added. The use of chemotherapy did not cause any treatment response delays in those animals that were treated with both chemotherapy and ionizing radiation.

Additional therapy

Additional therapy was offered to all patients after progressive disease was observed, regardless of the initial treatment. In 15 curatively treated cats it consisted either of surgery (n=7), chemotherapy (n=1), radiation therapy (n=1) or a combination of these treatments (n=6). In 6/17 cats with macroscopic disease subjected to coarse fractionated therapy additional therapy consisted of either a second round of radiation therapy (n=3), ancillary surgery (n=2) or chemotherapy (n=1). Two cats with no visible mass treated with coarse fractionation protocol received additional therapy consisting of a combination of these modalities.

Histopathological reassessment

Paraffin blocks from 55 of 73 cats were available for histopathological reassessment. In the remaining cases no paraffin blocks were available. The tumours were classified as fibrocytic (n=25), histiocytic (n=4) or mixed cell type (n=26) in dependence of the predominant cellular morphology (Figure 1). The detailed results of histological grading are reported in Table 1. For statistical analysis 27 cats (49%) were assigned to the low grade group (comprising histological grade I and II) and 28 animals (51%) to the high grade group (histological grade III). Multinucleated giant cells did not appear in histological grade I tumours and were detected in only 6/25 grade II tumours and in 20/28 grade III tumours.

Ki67-index

The mean number of cells counted to assess the Ki67-index was 1272 (range 711 - 3015). Ki67 could be evaluated in 52/55 cases. Three cases were excluded: in one

instance, too many cells stained positive for CD3 and CD79alpha preventing proper identification of tumour cells for counting. And in two other cases it was not possible to count a minimum of 700 cells. Of the 52 cases evaluated, 36 were curatively treated and 16 were treated with coarse fractionated radiotherapy. The percentage of Ki67 positive cells varied between 0% and 40%, with a mean of 14% (Figure 1). The Ki67-index did not correlate with grade, neither in a continuous form nor after grouping using any of the cut-offs (10%, 20%).

PFI and survival

The median follow-up time for all animals in this study was 21 months. The median follow-up time in the curative group was 25 months, while the group that received coarse fractionated therapy had a median follow-up of 10 months. For the censored animals in the curative group, the median follow-up time was 32 months and for the cats receiving coarse fractionated therapy, it was 12 months.

The main data related to the PFI are summarized in tables 3a and 3b and in figures 2 and 3. Curatively treated patients (n=46) had a median PFI of 37 months (95% CI 19 - 56 months). In this group, 63% (n=29) of the animals were progression free after one year (95% CI 49% - 78%) and 60% (n=28) after two years (95% CI 45% - 75%). Fifty percent (n=23) of the cats were tumour-free at the time of data analysis and were therefore censored. The median PFI of all cats receiving coarse fractionated therapy (n=27) was 10 months (95% CI 8 – 15 months). Seven of these 27 patients (26%) were censored. Cats with no visible mass receiving coarse fractionated therapy (n=10) had a significantly longer median PFI (20 months, 95% CI 9 – 30 months) than those with macroscopic disease (n=17, 4 months, 95% CI 2 – 5 months; P=0.01). In the curatively

treated group the Ki67-index had no significant impact on PFI with any of the predictors examined. In the group treated with coarse fractionated radiotherapy the Ki67-index showed no significant influence on PFI when examined in a continuous form, a tendency towards a longer PFI in cases with more than 10% positive tumour cells (P=0.0815) and a significantly longer PFI in cases with more than 20% positive tumour cells (P=0.015, multiple Cox-regression analysis). Differentiation of no visible mass/macroscopic disease was the only other predictor of PFI for cats with coarse fractionation suggested by the backward Cox-regression (P=0.002).

The main data related to survival are summarized in tables 3a and 3b and in figures 4 to 6. Curatively treated cats (n=46) displayed a median survival time of 43 months (95% CI 40 – 46 months). Eighty-six percent (n=39) of these animals were alive after one year (95% CI 76% - 96%), 71% (n=33) after two years (95% CI 56 – 85%), and 68% n=31) after three years (95% CI 53% - 83%). Twenty-eight of the 46 (61%) cats in this group were still alive at the time of data analysis or dead due to tumour-unrelated disease, and were therefore censored. The median survival time for cats undergoing coarse fractionated therapy (n=27) was 24 months (95% CI 4 – 43 months). Fifty-nine percent (n=17) of these animals were alive after one year (95% CI 39% - 79%). Of the 27 cats treated with coarse fractionated radiotherapy nine animals (33%) were censored. Patients with no visible mass receiving coarse fractionated therapy (n=10) lived significantly longer (median survival 30 months; 95% CI 21 – 40 months) than cats with macroscopic disease (n=17, median survival time of 7 months; 95% CI 5 – 10 months; P=0.025). However, survival in cats with macroscopic disease was significantly prolonged, when adjuvant chemotherapy was used (median survival 29 months, 95% CI

11 – 46 months; P=0.04; n=5). In cats with no visible mass additional benefit of chemotherapy over coarse fractionated radiotherapy alone could not be evaluated statistically. The Ki67-index did not correlate in any of the forms examined with survival neither in curatively treated patients nor in animals receiving coarse fractionated therapy.

Tumour margins had no significant impact on outcome. In curatively treated cats, there was no significant difference in outcome neither between cats with wide (n=22) margins and cats with clean but close (n=11) margins nor between cats with complete (n=33) and incomplete (n=6) excisions. The outcome in the cats with wide excisions (n=22, including the cat with surgery after radiation therapy) and the cats with clean but close and dirty margins (n=11 and n=6, respectively) did not significantly differ either. When the outcome in the curative group was compared with a group where "non-confirming" cats as the one cat that received chemotherapy (n=1), the cats with dirty margins (n=6), those with gross disease (n=3), and those with non-assessable margins (n=5) were removed, the 95% confidence intervals for median PFI and survival time overlapped between these two groups. This suggests that no signs of bias were introduced by these "non-confirming" cats. This was also true when the outcome in the curative group was compared with another group where different "non-confirming" cats were removed including the one cat that received chemotherapy (n=1), the cats with gross disease (n=3), the cats with non-assessable margins (n=5) and those with wide excisions (n=21). No significant difference was observed when comparing the outcome in cats with dirty margins in the curative group (n=6) versus in the coarse fractionated therapy group (n=7). Low animal numbers prevented statistical analysis of the margin status in regard

to outcome in cats with no visible mass treated with the coarse fractionation protocol. The number of previous surgeries did not influence the outcome in curatively treated cats. In contrast, an increasing number of previous surgeries was significantly associated with a shorter survival time in cats undergoing coarse fractionated radiotherapy (Coeff=0.41, P=0.03). Additional therapy did not influence survival in the curatively treated cats as indicated by a lack of difference between outcomes in cats with and without additional therapy. In contrast, additional therapy resulted in significantly prolonged survival in cats subjected to coarse fractionated therapy (n=8, P=0.001).

None of the further demographical, clinical and histological parameters tested correlated with outcome.

DISCUSSION

In this study cats treated with a curative intent had a median survival of 43 months and a median PFI of 37 months. The latter variable was longer in our collective than in two previous studies (14 months; 22 months)[18, 19] where radiation therapy was also applied after surgery. In a further previous study where radiation therapy was applied before surgery the PFI was shorter as well (20 months)[21]. Outcomes in our series were achieved with total doses of 45 or 48 Gy, which is considerably lower than those reported in the former two above mentioned studies (52 or 57 Gy)[18, 19]. This favourable outcome might be due to the large proportion of cats with complete surgical excision. However, in our study there was no significant difference in curatively treated cats between completely and incompletely excised tumours regarding survival and PFI. In contrast, previous investigations have shown that cats with complete surgical excision have a significant better outcome than those with incomplete excision[15, 21, 45]. A factor complicating the assessment of the prognostic significance of the tumour margins is that criteria for complete and incomplete excision were not defined[15, 45] or differed[21] from those of the present study. Although the data cannot be directly compared due to basic differences in the study designs, results of the present study support the notion that adding curative radiation after surgery improves outcome.

In this study cats treated with a coarse fractionation protocol had a median survival of 24 months and a PFI of 10 months. These times are somehow surprisingly long. To our knowledge, a coarse fractionation protocol has never before been reported for use in cats with vaccine-associated sarcoma. In our practice it turned out to meet a need of the owners because of its reduced costs and time requirements in contrast to the curative

protocol. It was primarily recommended for cats with incomplete excision because of their possible higher likelihood of recurrence and poor outcome[15, 21, 45]. The cats with no visible mass in this group had a median survival of 30 months and a median PFI of 20 months. In a previous study the median time to first event for preoperative curative radiation followed by an incomplete excision was 292 days (10 months)[21]. In another study, Bregazzi *et al*. irradiated microscopic remnants at the surgical site with curative intent and administered doxorubicin afterwards. The animals in that report reached a median PFI of 661 days (22 months)[19], which is similar to the figure found in our study (20 months). However, a smaller number of fractions and a lesser total dose were used in our patients compared to the Bregazzi study. In both studies (ours and Bregazzi's), radiation therapy was applied after surgery. Therefore, although the overall number of animals used in this study was low, our data suggest that a postsurgical coarse fractionation protocol may have a role in the management of cats with no visible mass and warrants further prospective evaluation.

In cats with gross disease and treated with a coarse fractionation protocol adjuvant chemotherapy had a positive effect on survival (the median survival increased from 5 months in cats without chemotherapy, n=12, to 29 months in cats receiving chemotherapy, n=5, P=0.04) but not on PFI. This survival data must be interpreted cautiously because of a possible influence of additional therapy that was applied after progressive disease. In addition, although chemotherapy was recommended for all cats receiving a coarse fractionation protocol, in this retrospective analysis it was not possible to determine how far clinical factors with influence on outcome (e.g. clinical signs or tumour size) played a role in the owner's decision to apply this treatment. For these reasons and since the number of patients was small these results need to be

confirmed with a higher number of cats. Previous studies showed either no[45] or only a modest[16] effect of chemotherapy alone in animals with nonresectable tumours. In the latter investigation, approximately 50% of the cats responded to chemotherapy and showed a median survival of 242 days (8 months). In many cats with macroscopic disease coarse fractionated therapy implies a palliative intent. The primary goal of palliation is not to provide long-term or definitive tumour control but to induce pain relief or to reduce dysfunction associated with the tumour in patients in which other factors such as advanced metastatic disease or a severe concurrent disease are likely to lead to their demise[53]. However, as suggested from the current study, a coarse fractionation protocol in combination with chemotherapy may improve outcome, thus providing an additional therapeutic option for such cats. The appropriate treatment (either coarse fractionation radiation protocol, or chemotherapy or both) should be chosen individually from case to case. In conclusion, our data suggest that a coarse fractionation protocol also should have a place in the management of macroscopic disease.

An important restriction applies to the survival data in general due to the retrospective character of this study. In fact, in many cases the cause of death was assessed by local veterinarians or by the owners themselves. Therefore, survival information must be interpreted cautiously. Similarly, the apparent lack of development of metastases during or after therapy in our collective may be a consequence of the lack of necropsy data. An absence of metastases is consistent with previous studies where vaccine-associated sarcomas were mainly locally invasive with no metastases or with low metastatic rates[19, 46]. In more recent reports, however, metastatic rates of 12%[18] and 21%[21, 55] have

been indicated. In a new study from 2008, Romanelli *et al.* cats with histologic grade 3 tumours were significantly more likely to develop metastasis than cats with grade 1 and 2 tumours[55].

In this study neither histologic grading nor its components taken individually were predictive of survival or PFI of cats diagnosed with vaccine-associated sarcomas. This is in contrast with human soft tissue sarcomas[48, 49, 50] where histologic grading is the most important prognostic factor. A previous study in cats where the same grading system was used also failed to demonstrate such a correlation in vaccine-associated sarcomas[41]. This was also the case in a further study by Davidson[15], who however used a different grading scheme. Our study confirms findings by Couto *et al.*[41] that multinucleated giant cells do not occur in grade I vaccine-associated sarcomas. However, and contrary to what is described in human patients with soft tissue sarcomas[51, 52], the presence of multinucleated giant cells does not appear to be useful to estimate prognosis in this tumour type.

In our study, Ki67-index had no impact on survival neither in cats treated with a curative nor with a coarse fractionation protocol. However, when the 20% cut-off was used in the multivariate analysis for cats undergoing coarse fractionated radiotherapy the PFI was significantly prolonged (P=0.015). Due to the heterogeneity of treatments (no visible mass vs. macroscopic disease / with vs. without chemotherapy) in this group of patients these results should be evaluated critically. In addition, it cannot be completely ruled out that our selection method for appropriate cores for Ki67 counts, which tended to exclude tumour regions with strong inflammation constituted a bias. However, a previous study has shown no differences in the Ki67 labelling index

between samples from the centre of the tumours and the periphery[41]. Most inflammatory infiltrates are found in the latter. In conclusion, the results of the Ki67 evaluation were rather unexpected and this topic will require further studies.

In summary, the results of the present study indicate that in vaccine-associated sarcomas the combination of surgery and subsequent radiation therapy is an effective option. Curatively treated cats displayed a median survival time of 43 months, while 86% of the cats were alive after one year (95% CI 76% - 96%), 71% after two years (95% CI 56 – 85%), and 68% after three years (95% CI 53% - 83%), and their median PFI was 37 months. A coarse fractionation protocol also appears to have a place in the management of vaccine-associated sarcomas. In this setting, factors predictive of a better outcome in our study include no visible mass as opposed to macroscopic disease, additional chemotherapy in cats with gross disease and a smaller number of surgeries performed before radiation therapy. The predictive value of the Ki67-index needs to be further clarified in feline vaccine-associated sarcomas.

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Figure legends and Tables (Figures available on request)

Table 1 - Criteria and results of histologic grading. Grade is the result of cumulative scoring by adding the individual scores for cellular differentiation, necrosis and mitotic rate. The results of this study are presented in bold (n=55).

Cellular Differentiation		**Necrosis**		**No. of mitotic figures per 10 hpf***	
Resembles normal adult mesenchymal tissue	**n= 0 (0%)**	None	**n= 8 (15%)**	0 to 9	**n= 19 (35%)**
Specific histologic type	**n= 40 (73%)**	< 50%	**n= 41 (75%)**	10 to 19	**n= 13 (24%)**
Undifferentiated	**n= 15 (27%)**	> 50%	**n= 6 (11%)**	≥ 20	**n= 23 (42%)**

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I cumulative score of 3 or 4: **n= 2 (3%)**

II cumulative score of 5 or 6: **n= 25 (46%)**

III cumulative score of 7 - 9: **n= 28 (51%)**

Table 2 - Clinical characteristics and adjuvant therapies in cats treated with curative or coarsely fractionated radiation therapy

RT protocol	**No. of cats with tumour appearance and surgery as indicated**		**No. of cats with tumour margins as indicated**		**No. of cats with adjuvant chemotherapy**
Curative (n=46)	no visible mass	43	wide	21	0
			clean but	11	1
			close	6	0
			dirty	5	0
			NA		
	gross disease	3			
	→surgery after RT	1	wide	1	0
Coarse (n=27)	no visible mass	10	dirty	7	6
			clean but	1	1
			close	2	1
			NA		
	gross disease	17			
	→no surgery	15	NR		
	→surgery after RT	2	wide	1	4
			clean but	1	1
			close		0

RT = radiation therapy; NA = not assessable; NR = not relevant

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